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PROTEIN COMPOUND CAPABLE OF INHIBITING TUMORAL GROWTH

Abstract:

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This invention relates to a substantially proteinaceous composition which is capable of inhibiting selectively the division of estrogen-sensitive tumoral cells and/or of exerting a cytotoxic activity on such cells. This invention also relates to a cell line isolated from a human liposarcoma, capable of dividing in a culture, which produces said composition, as well as to its culture medium has been conditioned, wherein said composition is present. This invention also relates to the various uses of said products in diagnostic and clinical applications. Data supplied from the esp@cenet database - Worldwide

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(57) Abstract

This invention relates to a substantially proteinaceous composition which is capable of inhibiting selectively the division of estrogen-sensitive tumoral cells and/or of exerting a cytotoxic activity on such cells. This invention also relates to a cell line isolated from a human liposarcoma, capable of dividing in a culture, which produces said composition, as well as to its culture medium has been conditioned, wherein said composition is present. This invention also relates to the various uses of said products in diagnostic and clinical applications.

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PROTEIN COMPOUND CAPABLE OF INHIBITING TUMORAL GROWTH

This invention relates to a substantially proteic composition which is capable of inhibiting selectively the division of tumoral cells sensitive to estrogens and/or of exerting a cytotoxic activity on said cells. This invention also relates to a cell line isolated from a human liposarcoma, capable of dividing in a culture, which line produces said composition, as well as to its culture conditioned medium in which said composition is present.

This invention is also concerned with the employments of said products in diagnostic and in clinical applications.

A large number of cell lines capable of growing in vitro and originally isolated from human tumors are already known from the prior art. Such lines are particularly useful for the study of their biochemical and physiological properties, as well as for the production of specific factors.

In spite of the large number of attempts made up to the present time, no cell lines are known from the prior art which have a differentiated phenotype that can be associated to that of the adipose cells or adipocytes, and no lines are known which are isolated from tumors belonging to the class of liposarcomas. Such cells would be particularly useful as they derive from the stromal tissue, a tissue which at the present time has been very little characterized and for which a controlling role has been suggested as regards some of the functions performed by the adjacent tissues. Indeed, in vivo, in the mammary gland in the presence of the hormone testosterone, adipose cells induce the regression of epithelial tissue [Kratochwill, K. Development and loss of androgen responsiveness in the embryonic rudiment of the mouse mammary gland.

Dev. Biol. 61: 358-365, 1977] so exerting paracrine action which possibly is mediated by one or more compounds released by said adipose cells [Kratochwill, K. 1987, in "Cellular and Molecular Biology of Mammary Cancer" (D. Medina, W. Kidwell, G. Heppner, e E. Anderson, eds.), pp. 1-8 Plenum, New York].

The investigations mentioned above put particularly into evidence the need for isolating and growing in vitro a cell line capable of performing the functions observed in vivo.

The Author of this invention has isolated and characterized for the first time a cell line which is capable of dividing in a culture, with a phenotype associable with that of adipose cells. Surprisingly, the cell line produces in the culture medium, denominated as conditioned medium, at least one compound that is capable of inhibiting selectively the division of epithelial tumoral cells deriving from epithelial tumors which are sensitive to estrogens.

By the expression "phenotype associable to the phenotype of adipose cells" it is meant the manifestation of at least one differentiated character which is proper of adipose cells, like the synthesis of lipids. By the expression "epithelial tumoral cells" it is meant tumoral cells derived from epithelial tumors, said cells being isolated or still present in the tumoral mass. By the expression "estrogen-sensitive epithelial tumors" it is meant tumors comprising cells with at least one receptor for a hormone belonging to the class of estrogens. By the expression "conditioned medium" it is meant the culture medium in the presence of which the cells are incubated under temperature, humidity and pH conditions suitable for growing the same, for at least 6 hours. By the "capability for inhibiting cell division" it is meant the capability of a compound for inhibiting at least 50 % of the cell growth in a culture, after at least 3 days of incu-

bation in the culture soil of said cells.

Accordingly, there is evidently the need for identifying and characterizing the compound/s capable of inhibiting selectively the division of tumoral cells which are derived from estrogen-sensitive epithelial tumors.

In a paper published recently [Mendelsohn M.E. et al., Proc. Natl. Acad. Sci. USA, 88, 11212-11216, 1991] it is disclosed that the "heat shock" p27 human protein (HSp27), already identified and sequenced by Hickey E. et al., Nucl. Acids Res., 14, 10 4127-4145 (1986), has the amino acid sequence of 199 aa. identical with that of a protein which is correlated to the estrogen-receptor and is called p29 [Coffer A.I. et al., Cancer Research 45, 3686-3693, 1990].

More recently [Carper S.W. et al., Nucl. Acids Res. 18, 6457, 1990], a cDNA clone obtained from the A549 cell line of human lung carcinoma has been sequenced. The nucleotide sequence of such clone codes for a protein of 205 amino acids in which the first 193 amino acids coincide with those of human HSp27 already known. In correspondence with the codon that codes for the amino acid residue in the position 194, the insertion of two cytosine residues has been detected, which determines a shift of the reading frame to the following stop codon in the positions 616-618 of the nucleotide sequence. The insertion thus causes a modification of the C-terminal sequence of the HSp27 protein that diverges for the last 5 amino acids (from the 195 position to the 199 position) and shows an extension of 6 more amino acids (from the position 200 to the position 205).

No inhibition activity of cell division of tumoral cells has been put into evidence in the prior art for any one of the proteins described.

Among the compounds produced and secreted from the LSA

line the Author of this invention has identified and characterized a compound, whose composition is substantially proteinaceous in nature, capable of inhibiting selectively the division of estrogen-sensitive tumoral epithelial cells and of exerting a cytotoxic activity on such cells. The Author has biochemically characterized said compound, which has been called p1LSA, and has determined its amino acid sequence, which has turned out to be made up of 205 amino acids. The Author has found surprisingly that said amino acid sequence is identical with the sequence of the human HSp27 protein already determined by Carper S.W. (ibid.) except for a single amino acid. The difference observed is in the amino acid 194 (arginine instead of proline) and is determined by a substitution of cytosine in the position 581 of the sequence that codes for the protein HSp27 with a guanine.

Accordingly, the Author has identified a new compound having an antineoplastic biological activity which is specific for estrogen-sensitive epithelial tumoral cells.

The Author has identified the nucleotide sequence that codes for the protein p1LSA and has determined its amino acid sequence.

Accordingly, it is an object of this invention a compound whose composition is substantially proteinaceous in nature, said compound being capable of inhibiting selectively the cellular division of estrogen-sensitive epithelial tumoral cells and/or of exerting a cytotoxic activity on said cells; said compound having a molecular weight in the range from 27 to 30 kDa; said compound also comprising an amino acid sequence from the amino acid 1 to the amino acid 193 which is substantially homologous to the corresponding sequence of human protein HSp27 (heat shock p27); or a fragment of said compound which is biologically active.

By the expression "substantially homologous amino acid sequences" it is meant in the scope of this invention to designate sequences with homologies in the range from 50 % to 100 % which do not give rise to a loss of biological activity of the protein. By "biological activity" it is meant the capability of inhibiting selectively the division of estrogen-sensitive epithelial tumoral cells (cytostatic activity) and/or of exerting a cytotoxic activity on said cells.

According to a preferred embodiment of this invention, said compound whose composition is substantially proteinaceous in nature comprises at its carboxyl end an amino acid sequence which is substantially homologous to the sequence:

GluAlaAlaLysSerAspGluThrAlaAlaLys-NH_2

Preferably said substantially proteinaceous compound comprises the following amino acid sequence:

MetThrGluArgArgValProPheSerLeuLeuArgGlyProSer 15
TrpAspProPheArgAspTrpTyrProHisSerArgLeuPheAsp 30
GlnAlaPheGlyLeuProArgLeuProGluGluTrpSerGlnTrp 45
LeuGlyGlySerSerTrpProGlyTyrValArgProLeuProPro 60
AlaAlaIleGluSerProAlaValAlaAlaProAlaTyrSerArg 75
AlaLeuSerArgGlnLeuSerSerGlyValSerGluIleArgHis 90
ThrAlaAspArgTrpArgValSerLeuAspValAsnHisPheAla 105
ProAspGluLeuThrValLysThrLysAspGlyValValGluIle 120
ThrGlyLysHisGluGluArgGlnAspGluHisGlyTyrIleSer 135
ArgCysPheThrArgLysTyrThrLeuProProGlyValAspPro 150
ThrGlnValSerSerSerLeuSerProGluGlyThrLeuThrVal 165
GluAlaProMetProLysLeuAlaThrGlnSerAsnGluIleThr 180
IleProValThrPheGluSerArgAlaGlnLeuGlyGlyArgGlu 195
AlaAlaLysSerAspGluThrAlaAlaLys 205.

According to a preferred embodiment of this invention, said substantially proteinaceous compound is produced by the LSA

cells (DSM ACC. N. 2029) preferably is secreted by said cells in the culture medium.

It is a further object of this invention a composition for pharmacological uses, preferably for antineoplastic applications, and even more preferably for the treatment of estrogen-sensitive epithelial tumors, that comprises the substantially proteinaceous compound of this invention or salts thereof which are physiologically acceptable.

It is a further object of this invention the employment of said substantially proteinaceous compound according to the invention or of its salts which are physiologically acceptable for the preparation of pharmacological compounds for anti-neoplastic treatment, preferably for the treatment of estrogen-sensitive epithelial tumors.

It is an object of this invention a nucleic acid comprising a nucleotide sequence that codes for the compound whose composition is substantially proteinaceous according to this invention, preferably said nucleic acid comprising the following nucleotide sequence:

```
ATGACCGAGCGCCGCGTCCCCTTCTCGCTCCTGCGGGGCCCCAGC  45
TGGGACCCCTTCCGCGACTGGTACCCGCATAGCCGCCTCTTCGAC  90
CAGGCCTTCGGGCTGCCCCGGCTGCCGGAGGAGTGGTCGCAGTGG 135
TTAGGCGGCAGCAGCTGGCCAGGCTACGTGCGCCCCCTGCCCCCC 180
GCCGCCATCGAGAGCCCCGCAGTGGCCGCGCCCGCCTACAGCCGC 225
GCGCTCAGCCGGCAACTCAGCAGCGGGGTCTCGGAGATCCGGCAC 270
ACTGCGGACCGCTGGCGCGTGTCCCTGGATGTCAACCACTTCGCC 315
CCGGACGAGCTGACGGTCAAGACCAAGGATGGCGTGGTGGAGATC 360
ACCGGTAAGCACGAGGAGCGGCAGGACGAGCATGGCTACATCTCC 405
CGGTGCTTCACGCGGAAATACACGCTGCCCCCCGGTGTGGACCCC 450
ACCCAAGTTTCCTCCTCCCTGTCCCCTGAGGGCACACTGACCGTG 495
GAGGCCCCCATGCCCAAGCTAGCCACGCAGTCCAACGAGATCACC 540
ATCCCAGTCACCTTCGAGTCGCGGGCCCAGCTTGGGGGCCGAGAA 585
GCTGCAAAATCCGATGAGACTGCCGCCAAGTAA 618
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A further object of this invention consists in plasmid or phage type vectors comprising the nucleotide sequences of this invention.

It is a further object of this invention a mammalian cell line capable of dividing in vitro, and having a phenotype associable to that of adipose cells, that produces also in its conditioned medium at least one compound capable of inhibiting selectively the division of tumoral cells, preferably epithelial tumoral cells, which even more preferably derive from estrogen-sensitive epithelial tumors.

According to a preferred embodiment of this invention, said cell line is isolated from a liposarcoma, preferably of human origin, and also more preferably such line is the LSA cell line deposited with the DSM with the accession number 2029. Again according to this invention, said cell line produces the substantially proteinaceous compound having the sequence disclosed above.

It is a further object of the invention a medium conditioned by the growth of cell lines herein disclosed and claimed.

Preferably said conditioned medium comprises at least one compound capable of inhibiting selectively the division of tumoral cells, preferably epithelial tumoral cells, and even more preferably cells deriving from estrogen-sensitive epithelial tumors, more preferably said compound being the protein compound disclosed according to this invention.

According to a preferred embodiment of the present invention, said medium is conditioned by the growth of the LSA cell line (DSM ACC. N. 2029).

It is another object of this invention the employment of said conditioned medium for producing pharmaceutical compositions for treating tumors, preferably epithelial tumors, and

even more preferably for the treatment of estrogen-sensitive epithelial tumors.

It is a further object of this invention the employment of said conditioned medium for the purification, identification and characterization of at least one compound capable of inhibiting selectively the division of tumoral cells, preferably of epithelial tumoral cells, and even more preferably of cells derived from estrogen-sensitive epithelial tumors.

This invention will be now disclosed by means of some examples that explain and illustrate the same but with no limitative purposes, and with reference to the enclosed figures wherein:

Figure 1 represents a growth curve of the LSA line both in the presence and in the absence of serum;

Figure 2a shows a cytofluorimetric analysis of human normal thymocytes;

Figure 2b shows a cytofluorimetric analysis of LSA cells:

Figure 3 shows an LSA-CM stimulation histogram of the incorporation of ^{3}[H]-thymidine in various cell lines, where 1 is the control sample, 2 is the LSA-CM at 1/4 dilution, 3 is the LSA-CM at 1/2 dilution, 4 is the LSA-CM undiluted, 5 is a control sample, 6 is the LSA-CM undiluted, 7 is the control sample, and 8 is the LSA-CM undiluted;

Figure 4 shows growth curves of the MCF-7 cell line, both in the presence and in the absence of LSA-CM:

Figure 5 represents growth curves of the ZR-75-1 cell line, both in the presence and in the absence of LSA-CM;

Figure 6 represents growth curves of the MDAMB-231 cell line, both in presence and in the absence of LSA-CM:

Figure 7 represents growth curves of the NMMG cell line in the absence and in the presence of LSA-CM;

Figure 8 represents growth curves of human cells from

ovaric carcinoma both in the absence and in the presence of LSA-CM;

Figure 9 represents a histogram of human cell growth from ovaric carcinoma both in the absence and in the presence of LSA-CM and/or of purified p1LSA.

Example 1

Isolation of the LSA line

A fragment of human liposarcoma of mixed lipoblastic-fibroblastic type is drawn in a sterile way from the leg of an adult woman, 65 years of age, by means of surgical techniques.

The fragment is disgregated mechanically by a chisel so as to obtain 1 mm fragments and then this material is treated with a CTC solution equivalent to 20 U/ml of collagenase (CLSP, Worthington, . U.K. .), 0,75 mg/ml of trypsin (1/300, Inc. Pharmaceuticals), 2 % chicken serum, heat-inactivated and dialyzed (GIBCO) in Hank soil without Ca^{++} and Mg^{++} ions (GIBCO) and Ham F12 soil with 5 % bovine serum (GIBCO) for 4 hours at 37°C and under continuous stirring, in order to obtain a suspension of single cells.

The cell suspension is diluted with the Ham F12 (GIBCO) culture medium supplemented with 5 % bovine serum (GIBCO), penicillin (31 µg/ml), streptomycin (50 µg/ml) and fungizone (2.5 µg/ml). The cells are plated at 200,000 cells/dish, 100 mm diameter (Costar).

A clone of cells which is capable of dividing in a culture and having the characteristics which are peculiar of the adipose cells has been obtained from ten dishes, said clone being called LSA and deposited with the DSM, accession number ACC 2029.

A growth curve of the LSA line is shown in Figure 1, in which the growth in the presence as well as in the absence

of serum is evident. The LSA line shows a plating efficiency of 90 % and a duplication time of 50-52 hours in the presence of serum and of 102 hours in the absence of serum. The medium is changed every 72 hours.

Example 2

Characterization of the LSA line

The DNA content of LSA cells has been analyzed by means of a cytofluorometer (Partec Pas II flow-cytometer). The cells are trypsinized then fixed with 70 % ethanol and dyed with a solution containing 5 µg/ml of ethidium bromide, 12.5 µg/ml of mitramycin and 1.5 mg $MgCl_2$ in 0.1 M Tris buffer, pH 7.5. The cell percentage in the various steps of the mitotic cycle is obtained as disclosed by Flintoff, W.E., Davidson, S.V., and Siminowitch, L. "Isolation and partial characterization of Methotrexate-Resistent phenotype from chinese hamster ovary cells" Somatic Cell Genet. 2:245-261, 1976; and Ambesi-Impiombato, F.S., Parks, L.A.M. and Coon H.G. Culture of hormone-dependent functional epithelial cells from rat thyroid. Proc. Natl. Acad. Sci. USA 77: 3455-3459, 1980. As shown in Figure 2b, the values are compared to those obtained with normal human thymocytes as ploidy standard. The value of 1 has been assigned to the G1-GO peak of thymocytes. Under the same conditions the G1-GO peak of the LSA cells has a value of 1.79, which is indicative of a DNA content which is almost tetraploid. Also the DNA content/cell is almost twice as much as that obtained with thymocytes as shown in Figure 2a.

The distribution of cells in the various steps of the cell cycle of an LSA cell population in the phase of logarithmic growth is shown in the following Table 1.

Table 1

Phase	Cell percentage
G1/GO	59

S	27
G2/M	14

The presence of cells in the phase S confirms that the population is in the active growth phase.

The counting of the chromosomes has put into evidence a number of chromosomes/cell between 80 and 90.

The LSA cells as observed under an optical microscope look like elongated bodies with abudant cytoplasm. Under the electronic microscope, the cell nucleus contains many nucleoles and some organelles are present, localized in the perinuclear region. The cells are homogeneous to each other, the mitochondria are relatively abudant and the cytoplasmic vesicles are constant both in number and sizes. The most evident feature is the presence of vacuolar areas inside the cytoplasm, said areas being electron-reflecting and typical of zones containing high amounts of lipids, which is removed as a result of subjecting the cells to a treatment for their preparation for observation under the electron microscope. Moreover, a well developed Golgi apparatus is also present.

In order to estimate the content of lipids, the LSA cells are plated on culture slides of the Lab-Teck (NUNC) and are grown by semi-confluence. The cells are washed and fixed for 6 minutes with 4 % paraformaldehyde and 15 % picric acid saturated in PBS (phosphate saline buffer). The cells are washed twice with distilled water, then they are dyed with an Oil Red O solution for 5 minutes, then they are washed twice with PBS and dyed with 1 % toluidine blue for 10 seconds.

If cells have been grown in the presence of 5 % bovine serum, lipids are dispersed throughout the cytoplasm, with round vesicles. The build up of lipids is not present in cells grown in a medium without serum, but it is stimulated by incubating cells with methylxanthine/desamethasone for 48 hours

and with insulin for a further period of 48 hours.

Example 3

Effects of conditioned medium by LSA (LSA-CM) cells on growth of different cell lines in vitro

LSA cells have been grown up to 80 % confluence on 100 mm plates (Costar) on F12 medium with 5% bovine serum. The cell monolayers are washed with PBS and incubated with F12 medium without serum. After 24 hours, the medium is replaced with fresh medium the conditioned medium is collected every 6-24 hours for the successive 30 days. The cells go on growing and they show structurally and functionally viable under phase-contrast microscope and after dying with Trypan Blue.

The medium conditioned by LSA cells is capable of stimulating the growth of normal epithelial cells like CHO (CCL-61), FRTL-5 (CRL-1468) and NIH-3T3 (ECACC-87100803) grown for 24 hours in the absence of serum, as put into evidence by an ^{3}H-thymidine incorporation test shown in Figure 3. Contrarily to the preceding results, the medium conditioned by LSA cells shows an inhibition effect of growth and a cytolytic activity when it is analyzed with in vitro tumoral cells containing receptors for estrogens. The cells employed are:

MCF-7 cells obtained from a human mammary carcinoma (Land, H. et al., Science 222:778 (1980), ECACC-86012803) that possess receptors for estrogens (Soule, H.D. et al., Natl. Cancer Inst. 51, 1409-1413 (1973)), androgens (Horowitz, K.B. et al., Steroids 26, 785-795 (1975)), insulin and triodothyronine (MacGrath, C.M. Cancer Res., 43: 1355-1360 (1983);

the ZR-75-1 (CRL-1504) cells obtained from a human mammary carcinoma, said cells being positive for estrogen receptors;

the MDAMB-231 (ATCC-HTB26) cells obtained froma a human mammary carcinoma, but that are negative for estrogen recep-

tors;

the NMMG (Burker, R.E. et al., Cancer Res., 38, 3769-3773 (1978), CRL-1636) cells, obtained from a mouse mammary epithelium, said cells being slightly positive for estrogen receptors;

a human cell line of ovaric carcinoma obtained from a woman suffering from peritoneal effusion due to ovaric carcinoma metastasis, said line being positive for estrogen receptor.

The growth curves of the cell lines mentioned herein have been obtained by plating 100,000 cells/dish of 100 mm diameter in 10 ml of DMEM (GIBCO) medium with 10 % bovine fetal serum (FCS, GIBCO). 2 ml of LSA-CM have been added to a set of dishes. .

Figure 4 shows growth curves of the MCF-7 line in which the inhibition effect of LSA-CM is evident. In particular, after a 4 day treatment, the removal of LSA-CM cannot re-establish the proliferative capability of MCF-7 cells because of a cytolytic effect.

Figure 5 shows growth curves of the 7R-75-1 line in the presence and in the absence of LSA-CM. The presence of LSA-CM gives rise to a strong inhibition of growth without any cytolytic effect.

Figure 6 and 7 show the growth curves of MDAMB-231 cells and of normal epithelial NMMG cells, in which the absence of growth inhibition due to LSA-CM is evident. Such cells do not show estrogen receptors.

Figure 8 shows that the LSA-CM conditioned medium has a cytotoxic effect on the cells of human ovaric carcinoma, even after just 24 hours of incubation.

The LSA-CM effect on the incorporation of ^{3}H-thymidine in different cell lines is shown in the following Table 2.

Table 2

Effect of LSA-CM on ^{3}H-thymidine incorporation in different cell lines

Cell lines	No effect	Stimulation	Inhibition
MCF7			+
ZR-75-1			+
NMMG	+		
FRTL-5		+	
CHO		+	
NIH-3T3		+	

Example 4

Effect of LSA-CM on animals

20 Balb/c fc3H mice (Charles River) which are affected by the Bittner virus are selected for the presence of an evident tumoral mass, an estrogen-dependent mammary carcinoma, which is induced by the virus itself.

After a peritumoral injection practised every day of 200 µl of LSA-CM for 15 days, the inhibition of growth and the necrosis of the tumoral mass are observed in 80 % of the mice treated.

Example 5

Isolation of the p1LSA protein from LSA-CM

The LSA-CM medium is concentrated 100 x, then it is subjected to dialysis against a isotonic phosphate buffer at pH 7.4 and then to gel filtration on P6 resin (Pharmacia). The fractions eluted are tested for their capability of inhibiting selectively the growth of MCF-7 cells. One only fraction shows said biological activity. A sample of said fraction is separated by electrophoresis on a polyacrylamide gel in non-denaturating conditions, identifying a protein of about 29 kDa which is called p1LSA. Said protein is electro-

eluted from the gel and employed for the production of polyclonal antibodies as disclosed in the following and for the sequence analysis.

The protein sequence is shown in the following Table 3.

As shown in Figure 9, the purified p1LSA protein has an evident cytotoxic effect on the growth of human ovaric tumoral cells.

Table 3

Amino acid sequence of the p1LSA protein and corresponding coding sequence

```
ATGACCGAGCGCCGCGTCCCCTTCTCGCTCCTGCGGGGCCCCAGC   45
MetThrGluArgArgValProPheSerLeuLeuArgGlyProSer   15

TGGGACCCCTTCCGCGACTGGTACCCGCATAGCCGCCTCTTCGAC   90
TrpAspProPheArgAspTrpTyrProHisSerArgLeuPheAsp   30

CAGGCCTTCGGGCTGCCCCGGCTGCCGGAGGAGTGGTCGCAGTGG  135
GlnAlaPheGlyLeuProArgLeuProGluGluTrpSerGlnTrp   45

TTAGGCGGCAGCAGCTGGCCAGGCTACGTGCGCCCCCTGCCCCCC  180
LeuGlyGlySerSerTrpProGlyTyrValArgProLeuProPro   60

GCCGCCATCGAGAGCCCCGCAGTGGCCGCGCCCGCCTACAGCCGC  225
AlaAlaIleGluSerProAlaValAlaAlaProAlaTyrSerArg   75

GCGCTCAGCCGGCAACTCAGCAGCGGGGTCTCGGAGATCCGGCAC  270
AlaLeuSerArgGlnLeuSerSerGlyValSerGluIleArgHis   90

ACTGCGGACCGCTGGCGCGTGTCCCTGGATGTCAACCACTTCGCC  315
ThrAlaAspArgTrpArgValSerLeuAspValAsnHisPheAla  105

CCGGACGAGCTGACGGTCAAGACCAAGGATGGCGTGGTGGAGATC  360
ProAspGluLeuThrValLysThrLysAspGlyValValGluIle  120

ACCGGTAAGCACGAGGAGCGGCAGGACGAGCATGGCTACATCTCC  405
ThrGlyLysHisGluGluArgGlnAspGluHisGlyTyrIleSer  135

CGGTGCTTCACGCGGAAATACACGCTGCCCCCCGGTGTGGACCCC  450
ArgCysPheThrArgLysTyrThrLeuProProGlyValAspPro  150
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ACCCAAGTTTCCTCCTCCCTGTCCCCTGAGGGCACACTGACCGTG 495
ThrGlnValSerSerSerLeuSerProGluGlyThrLeuThrVal 165

GAGGCCCCCATGCCCAAGCTAGCCACGCAGTCCAACGAGATCACC 540
GluAlaProMetProLysLeuAlaThrGlnSerAsnGluIleThr 180

ATCCCAGTCACCTTCGAGTCGCGGGCCCAGCTTGGGGGCCGAGAA 585
IleProValThrPheGluSerArgAlaGlnLeuGlyGlyArgGlu 195

GCTGCAAAATCCGATGAGACTGCCGCCAAGTAA 618
AlaAlaLysSerAspGluThrAlaAlaLys 205
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From a comparison with the sequences deposited with data banks, the sequence turns out to be identical with that corresponding to the human HSp27 protein described by Carper S.W. mentioned above, except for just one amino acid. The difference consists in a substitution of the amino acid in the position 194, said amino acid turning out to be arginine instead of proline.

Example 6

Production of anti-p1LSA polyclonal antibodies, and inhibition of the activity of the p1LSA protein

100 µg of p1LSA protein electroeluted from a 10 % polyacrylamide gel is dissolved in 1 ml of PBS (phosphate saline buffer) and mixed with 1 ml of a complete Freund adjuvant. The mixture is injected through intramuscolar injection in a rabbit four times at 10 day intervals. 30 ml of immune serum are drawn from rabbits every other day for 20 days. The immunoglobulin (Ig) fraction is purified by the Mab Trap G (Pharmacia) according to the procedure recommended by the producer.

Immunoprecipitation and immunoblotting tests show that immunoglobulins react in a specific way with the p1LSA protein from LSA-CM.

A pre-incubation of 100 µl of LSA-CM with 10 µl of an Ig solution at 1 mg/ml for 30 minutes at 37°C stops the activity of inhibition of tumoral epithelial cell (MCF-7) division, restoring an ^{3}H-thymidine incorporation and growth curves similar to the untreated control sample. The LSA-CM activity turns out to be unaltered after incubation with pre-immune serum.

Such experimental data show that the p1LSA protein isolated and purified according to the procedures disclosed in Example 5 and having the sequence disclosed in Table 3 is actually the active principle responsible for the activity of inhibition of epithelial cell division disclosed herein.

Example 7

Nucleotide sequence coding for the p1LSA protein

The nucleotide sequence coding for the p1LSA protein is determined by purifying the RNA polyA^{+} from LSA cells following standard methods and employing the following primers for the PCR reaction:

oligo 5': CGGAATTCTGAGCAGACGTCCAGAG
(EcoRI)

oligo 3': CGGAATTCCGGGCTAAGGCTTTACTTG
(EcoRI)

The sequences are deduced respectively from the sequences at the positions 5' and 3' not translated of the gene that codes for the HSp27.

The amplification products are cloned directly in the EcoRI site of the vectors pGEM4z (Promega) and sequenced by means of the dideoxy method. The complete sequence of the coding portion is shown in the preceding Table 3.

This invention has been disclosed with reference to some

preferred embodiments of the same but it is to be understood that modifications and/or changes can be introduced by those who are skilled in the art without departing from the spirit and scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Ist. Naz. per Studio e Cura dei Tumori Fond. G. Pascale
(B) STREET: Via M. Semmola
(C) CITY: Naples
(E) COUNTRY: Italy
(F) POSTAL CODE (ZIP): 80131

(ii) TITLE OF INVENTION: Protein compound, coding nucleotide sequences, producing cell line and uses thereof

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: IT RM92/A000161
(B) FILING DATE: 09-MAR-1992

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: IT RM92/A000716
(B) FILING DATE: 30-SEP-1992

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 618 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(C) INDIVIDUAL ISOLATE: patient with liposarcoma
(F) TISSUE TYPE: Adipose tissue
(G) CELL TYPE: Adipocyte
(H) CELL LINE: LSA cell line deposited at DSM N. ACC2029

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: cDNA library from poly A+ RNA
(B) CLONE: p1LSA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..618

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
ATG ACC GAG CGC CGC GTC CCC TTC TCG CTC CTG CGG GGC CCC AGC TGG          48
Met Thr Glu Arg Arg Val Pro Phe Ser Leu Leu Arg Gly Pro Ser Trp
 1               5                  10                  15

GAC CCC TTC CGC GAC TGG TAC CCG CAT AGC CGC CTC TTC GAC CAG GCC          96
Asp Pro Phe Arg Asp Trp Tyr Pro His Ser Arg Leu Phe Asp Gln Ala
            20                  25                  30

TTC GGG CTG CCC CGG CTG CCG GAG GAG TGG TCG CAG TGG TTA GGC GGC         144
Phe Gly Leu Pro Arg Leu Pro Glu Glu Trp Ser Gln Trp Leu Gly Gly
        35                  40                  45

AGC AGC TGG CCA GGC TAC GTG CGC CCC CTG CCC CCC GCC GCC ATC GAG         192
Ser Ser Trp Pro Gly Tyr Val Arg Pro Leu Pro Pro Ala Ala Ile Glu
    50                  55                  60

AGC CCC GCA GTG GCC GCG CCC GCC TAC AGC CGC GCG CTC AGC CGG CAA         240
Ser Pro Ala Val Ala Ala Pro Ala Tyr Ser Arg Ala Leu Ser Arg Gln
65                  70                  75                  80

CTC AGC AGC GGG GTC TCG GAG ATC CGG CAC ACT GCG GAC CGC TGG CGC         288
Leu Ser Ser Gly Val Ser Glu Ile Arg His Thr Ala Asp Arg Trp Arg
                85                  90                  95

GTG TCC CTG GAT GTC AAC CAC TTC GCC CCG GAC GAG CTG ACG GTC AAG         336
Val Ser Leu Asp Val Asn His Phe Ala Pro Asp Glu Leu Thr Val Lys
            100                 105                 110

ACC AAG GAT GGC GTG GTG GAG ATC ACC GGT AAG CAC GAG GAG CGG CAG         384
Thr Lys Asp Gly Val Val Glu Ile Thr Gly Lys His Glu Glu Arg Gln
        115                 120                 125

GAC GAG CAT GGC TAC ATC TCC CGG TGC TTC ACG CGG AAA TAC ACG CTG         432
Asp Glu His Gly Tyr Ile Ser Arg Cys Phe Thr Arg Lys Tyr Thr Leu
    130                 135                 140

CCC CCC GGT GTG GAC CCC ACC CAA GTT TCC TCC TCC CTG TCC CCT GAG         480
Pro Pro Gly Val Asp Pro Thr Gln Val Ser Ser Ser Leu Ser Pro Glu
145                 150                 155                 160

GGC ACA CTG ACC GTG GAG GCC CCC ATG CCC AAG CTA GCC ACG CAG TCC         528
Gly Thr Leu Thr Val Glu Ala Pro Met Pro Lys Leu Ala Thr Gln Ser
                165                 170                 175

AAC GAG ATC ACC ATC CCA GTC ACC TTC GAG TCG CGG GCC CAG CTT GGG         576
Asn Glu Ile Thr Ile Pro Val Thr Phe Glu Ser Arg Ala Gln Leu Gly
            180                 185                 190

GGC CGA GAA GCT GCA AAA TCC GAT GAG ACT GCC GCC AAG TA               618
Gly Arg Glu Ala Ala Lys Ser Asp Glu Thr Ala Ala Lys
        195                 200                 205
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 205 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Thr Glu Arg Arg Val Pro Phe Ser Leu Leu Arg Gly Pro Ser Trp
1 5 10 15

Asp Pro Phe Arg Asp Trp Tyr Pro His Ser Arg Leu Phe Asp Gln Ala
20 25 30

Phe Gly Leu Pro Arg Leu Pro Glu Glu Trp Ser Gln Trp Leu Gly Gly
35 40 45

Ser Ser Trp Pro Gly Tyr Val Arg Pro Leu Pro Pro Ala Ala Ile Glu
50 55 60

Ser Pro Ala Val Ala Ala Pro Ala Tyr Ser Arg Ala Leu Ser Arg Gln
65 70 75 80

Leu Ser Ser Gly Val Ser Glu Ile Arg His Thr Ala Asp Arg Trp Arg
85 90 95

Val Ser Leu Asp Val Asn His Phe Ala Pro Asp Glu Leu Thr Val Lys
100 105 110

Thr Lys Asp Gly Val Val Glu Ile Thr Gly Lys His Glu Glu Arg Gln
115 120 125

Asp Glu His Gly Tyr Ile Ser Arg Cys Phe Thr Arg Lys Tyr Thr Leu
130 135 140

Pro Pro Gly Val Asp Pro Thr Gln Val Ser Ser Ser Leu Ser Pro Glu
145 150 155 160

Gly Thr Leu Thr Val Glu Ala Pro Met Pro Lys Leu Ala Thr Gln Ser
165 170 175

Asn Glu Ile Thr Ile Pro Val Thr Phe Glu Ser Arg Ala Gln Leu Gly
180 185 190

Gly Arg Glu Ala Ala Lys Ser Asp Glu Thr Ala Ala Lys
195 200 205

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 6; 7; 9; , line 1; 29; 30;

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution DSM - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH -

Address of depositary institution (*including postal code and country*)
Mascheroder Weg 1 B
D-3300 BRAUNSCHWEIG - GERMANY

Date of deposit	Accession Number
4th FEBRUARY, 1992	ACC2029

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*) — This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

The indications listed below will be submitted to the International Bureau later (*specify the general nature of the indications e.g., "Accession Number of Deposit"*)

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

CLAIMS

1. A substantially proteinaceous compound, characterized in that it is capable of inhibiting selectively the cellular division of estrogen-sensitive epithelial tumoral cells, and/or of exerting a cytotoxic activity on such cells; and characterized in that it has a molecular weight in the range between 27 and 30 kDa: it comprises an amino acid sequence from the amino acid 1 to the amino acid 193 which is substantially homologous to the corresponding sequence of human HSp27 (heat shock p27) protein; or a biologically active fragment of said compound.

2. A substantially proteinaceous compound according to claim 1, said compound comprising at its carboxyl end an amino acid sequence which is homologous to the sequence:

GluAlaAlaLysSerAspGluThrAlaAlaLys-NH_2.

3. A substantially proteinaceous compound according to claim 2, such compound comprising an amino acid sequence which is substantially homologous to the following amino acid sequence:

MetThrGluArgArgValProPheSerLeuLeuArgGlyProSer 15
TrpAspProPheArgAspTrpTyrProHisSerArgLeuPheAsp 30
GlnAlaPheGlyLeuProArgLeuProGluGluTrpSerGlnTrp 45
LeuGlyGlySerSerTrpProGlyTyrValArgProLeuProPro 60
AlaAlaIleGluSerProAlaValAlaAlaProAlaTyrSerArg 75
AlaLeuSerArgGlnLeuSerSerGlyValSerGluIleArgHis 90
ThrAlaAspArgTrpArgValSerLeuAspValAsnHisPheAla 105
ProAspGluLeuThrValLysThrLysAspGlyValValGluIle 120
ThrGlyLysHisGluGluArgGlnAspGluHisGlyTyrIleSer 135
ArgCysPheThrArgLysTyrThrLeuProProGlyValAspPro 150
ThrGlnValSerSerSerLeuSerProGluGlyThrLeuThrVal 165

GluAlaProMetProLysLeuAlaThrGlnSerAsnGluIleThr 180
IleProValThrPheGluSerArgAlaGlnLeuGlyGlyArgGlu 195
AlaAlaLysSerAspGluThrAlaAlaLys 205.

4. A substantially proteinaceous compound according to any one of the preceding claims, which is produced by LSA cells (DSM ACC. N. 2029), which compound is preferably secreted by said cells in the culture medium.

5. A composition for pharmacological use, preferably for use as anti-neoplastic, comprising the substantially proteinaceous compound according to any one of the preceding claims, or physiologically acceptable salts thereof.

6. A composition according to claim 5 to be employed for treatment of estrogen-sensitive epithelial tumors.

7. Use of the substantially proteinaceous compound according to any one of the preceding claims or of its salts physiologically acceptable, for the preparation of pharmacological compositions for antineoplastic treatment, preferably of estrogen-sensitive epithelial tumors.

8. A nucleic acid comprising a nucleotide sequence coding for said substantially proteinaceous compound according to any one of the preceding claims.

9. A nucleic acid according to claim 8 comprising the following nucleotide sequence:

ATGACCGAGCGCCGCGTCCCCTTCTCGCTCCTGCGGGGCCCCAGC 45
TGGGACCCCTTCCGCGACTGGTACCCGCATAGCCGCCTCTTCGAC 90
CAGGCCTTCGGGCTGCCCCGGCTGCCGGAGGAGTGGTCGCAGTGG 135
TTAGGCGGCAGCAGCTGGCCAGGCTACGTGCGCCCCCTGCCCCCC 180
GCCGCCATCGAGAGCCCCGCAGTGGCCGCGCCCGCCTACAGCCGC 225
GCGCTCAGCCGGCAACTCAGCAGCGGGGTCTCGGAGATCCGGCAC 270
ACTGCGGACCGCTGGCGCGTGTCCCTGGATGTCAACCACTTCGCC 315
CCGGACGAGCTGACGGTCAAGACCAAGGATGGCGTGGTGGAGATC 360
ACCGGTAAGCACGAGGAGCGGCAGGACGAGCATGGCTACATCTCC 405

CGGTGCTTCACGCGGAAATACACGCTGCCCCCCGGTGTGGACCCC 450
ACCCAAGTTTCCTCCTCCCTGTCCCCTGAGGGCACACTGACCGTG 495
GAGGCCCCCATGCCCAAGCTAGCCACGCAGTCCAACGAGATCACC 540
ATCCCAGTCACCTTCGAGTCGCGGGCCCAGCTTGGGGGCCGAGAA 585
GCTGCAAAATCCGATGAGACTGCCGCCAAGTAA 618

10. Plasmid or phage vectors comprising the nucleotide sequences according to any one of the preceding claims 8 or 9.

11. A mammalian cell line capable of dividing in vitro, having a phenotype associable to that of adipose cells, which produces in its culture medium, or conditioned medium, at least one compound according to any one of the preceding claims 1-4 which is capable of inhibiting selectively the division of tumoral cells.

12. A mammalian cell line according to claim 11 wherein said tumoral cells comprise epthelial tumoral cells.

13. A mammalian cell line according to claim 12 wherein said epithelial tumoral cells derive from estrogen-sensitive epithelial tumors.

14. A mammalian cell line according to any one of the preceding claims 11-13 and capable of dividing in vitro both in the presence and in the absence of serum in the culture medium.

15. A mammalian cell line according to any one of the preceding claims 11-14 wherein said cell line is isolated from a liposarcoma.

16. A mammalian cell line according to claim 15 wherein said cell line is isolated from a human liposarcoma.

17. A mammalian cell line according to claim 16, wherein said cell line is the LSA line (DSM ACC. N. 2029).

18. A medium that has been conditioned by the growth of a cell line according to any one of the preceding claims 11-17.

19. A medium that has been conditioned according to claim 18, said medium comprising at least one compound capable of inhibiting selectively the division of tumoral cells.

20. A medium that has been conditioned according to claim 19, wherein said tumoral cells are epithelial tumoral cells.

21. A medium that has been conditioned according to claim 20 wherein said epithelial tumoral cells derive from estrogen-sensitive epithelial tumors.

22. A medium that has been conditioned according to claim 21, wherein said cell line is the LSA line (DSM ACC.N. 2029).

23. Use of the conditioned medium according to any one of the preceding claims 18-22 for the production of pharmaceutical compositions for treatment of tumors.

24. Use of the conditioned medium according to claim 23 wherein said tumors are epithelial tumors.

25. Use of the conditioned medium according to claim 24 wherein said epithelial tumors are estrogen-sensitive.

26. Use of the conditioned medium according to any one of the preceding claims 18-22 for the purification, identification and characterization of at least one compound which is capable of inhibiting selectively the division of tumoral cells.

27. Use of the conditioned medium according to claim 26, wherein said tumoral cells are epithelial cells.

28. Use of the conditioned medium according to claim 27 wherein said cells are derived from estrogen-sensitive epithelial tumors.

FIG. 1

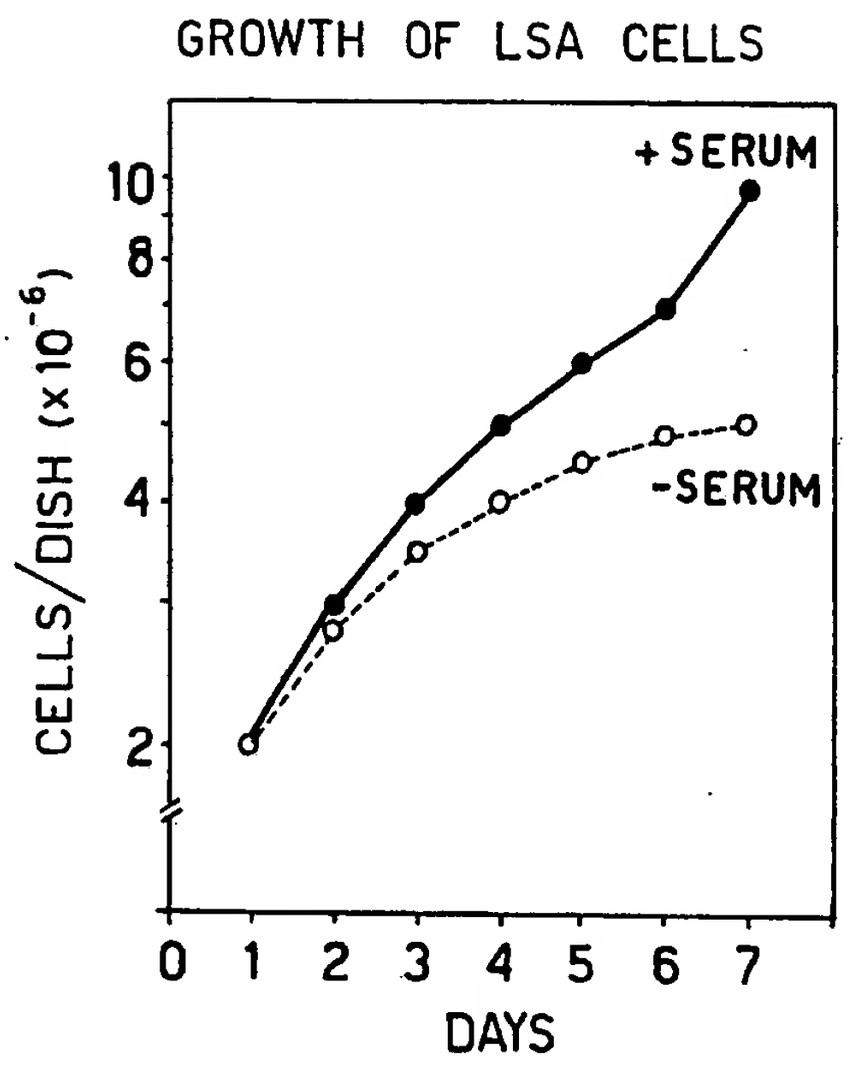

GROWTH OF LSA CELLS
+SERUM
-SERUM
CELLS/DISH (x 10⁻⁶)
10
8
6
4
2
0 1 2 3 4 5 6 7
DAYS

FIG. 5

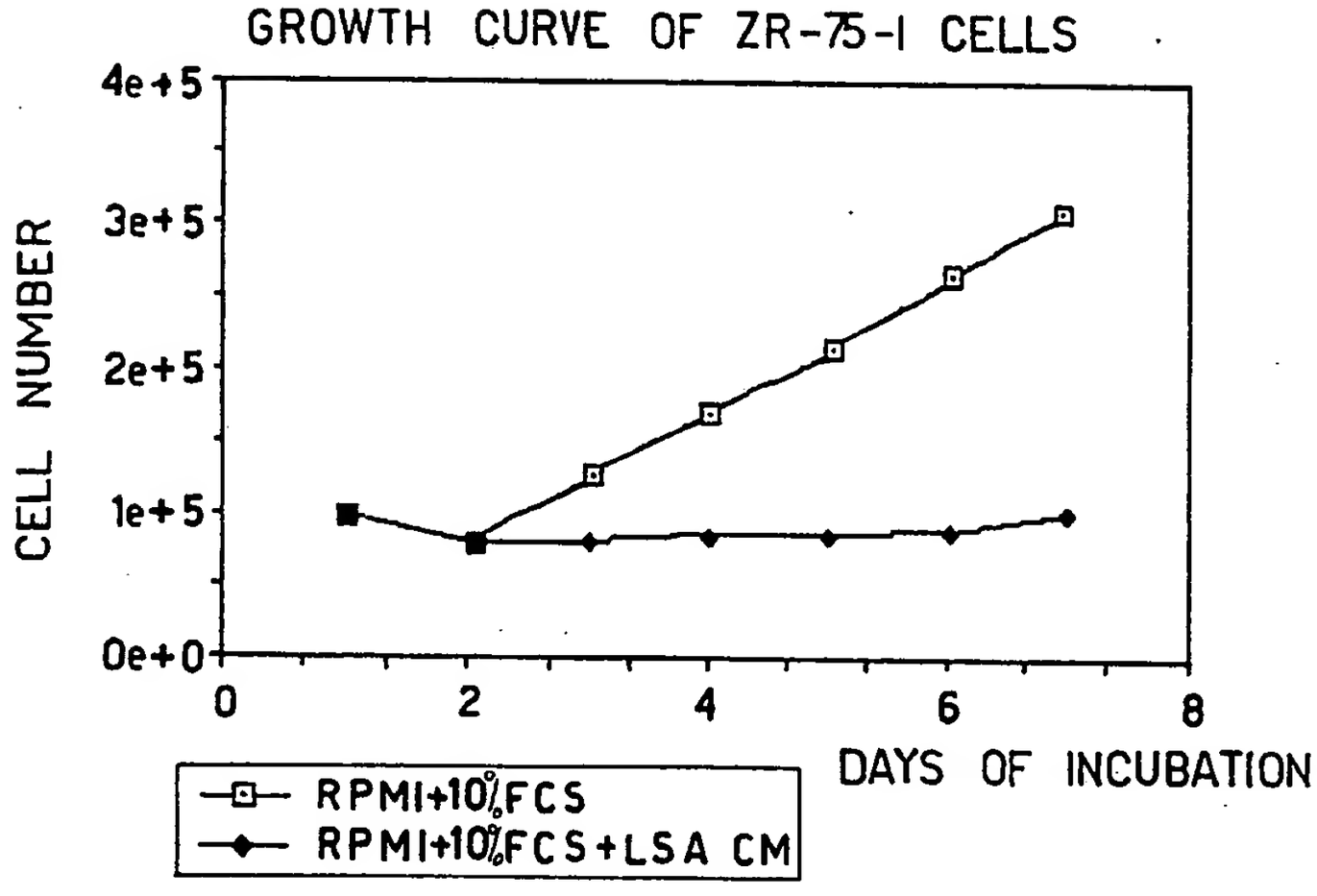

GROWTH CURVE OF ZR-75-1 CELLS
CELL NUMBER
4e+5
3e+5
2e+5
1e+5
0e+0
0
2
4
6
8
DAYS OF INCUBATION
RPMI+10%FCS
RPMI+10%FCS+LSA CM

FIG. 2A

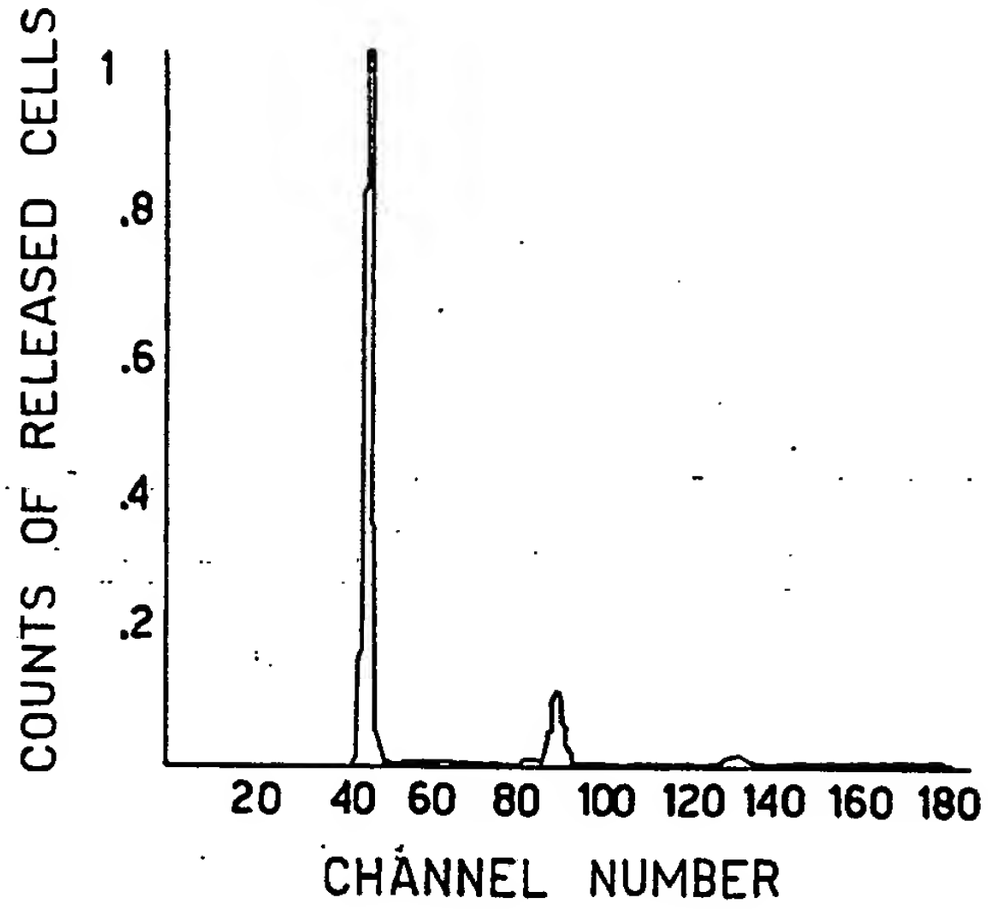

COUNTS OF RELEASED CELLS
1
.8
.6
.4
.2
20 40 60 80 100 120 140 160 180
CHANNEL NUMBER

FIG. 2B

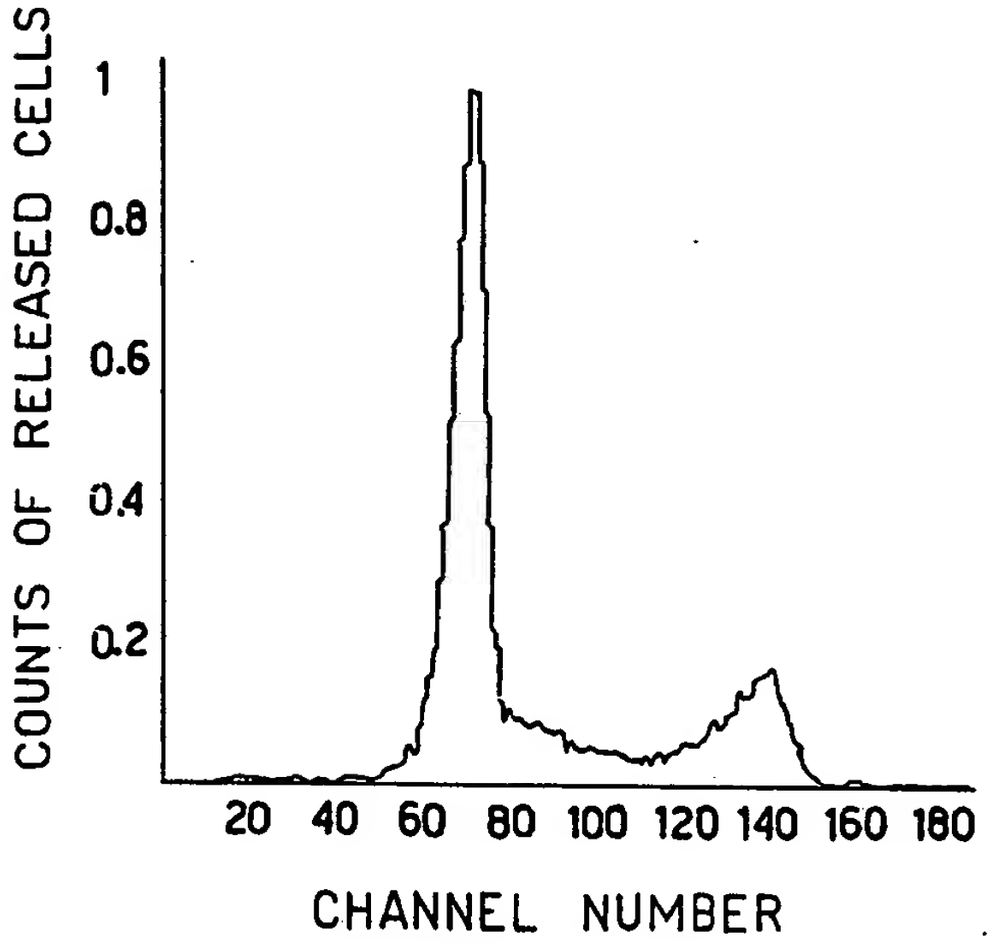

COUNTS OF RELEASED CELLS
1
0.8
0.6
0.4
0.2
20 40 60 80 100 120 140 160 180
CHANNEL NUMBER

FIG. 3

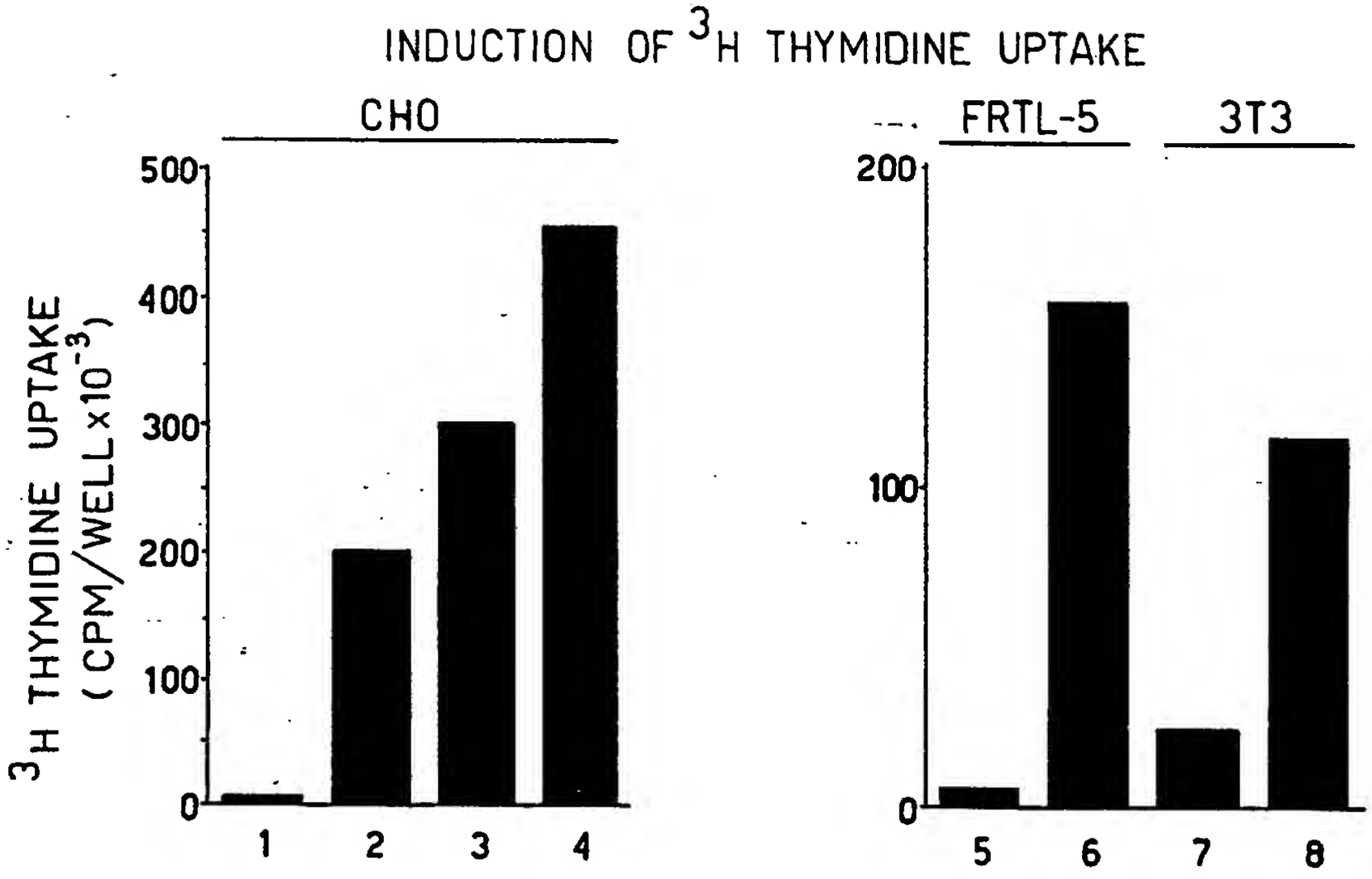

INDUCTION OF 3H THYMIDINE UPTAKE
CHO
FRTL-5
3T3
3H THYMIDINE UPTAKE (CPM/WELL x 10-3)
500
400
300
200
100
0
200
100
0
1
2
3
4
5
6
7
8

FIG. 4

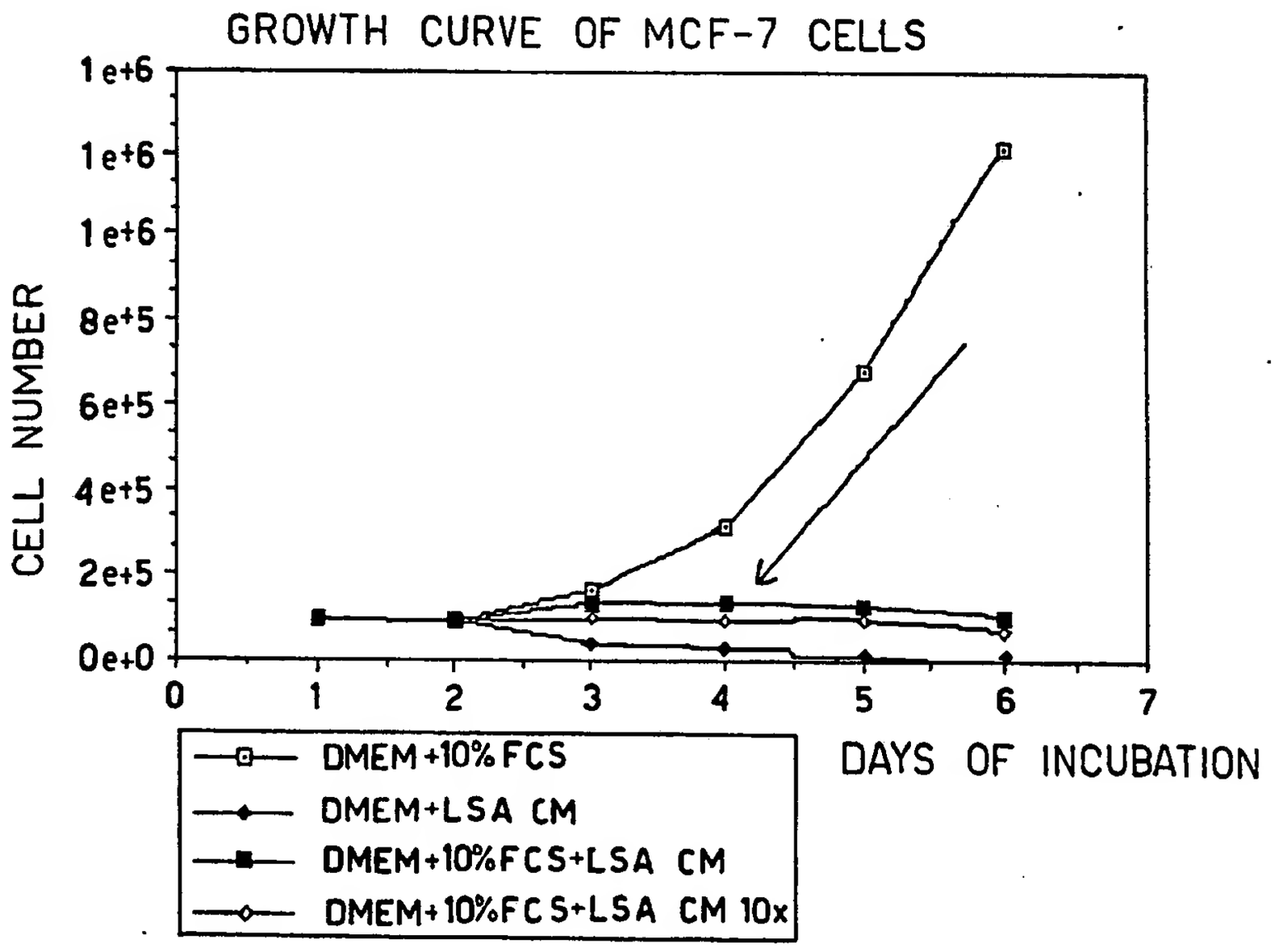

GROWTH CURVE OF MCF-7 CELLS
CELL NUMBER
1e+6
1e+6
1e+6
8e+5
6e+5
4e+5
2e+5
0e+0
0
1
2
3
4
5
6
7
DAYS OF INCUBATION
DMEM+10% FCS
DMEM+LSA CM
DMEM+10%FCS+LSA CM
DMEM+10%FCS+LSA CM 10x

FIG. 6

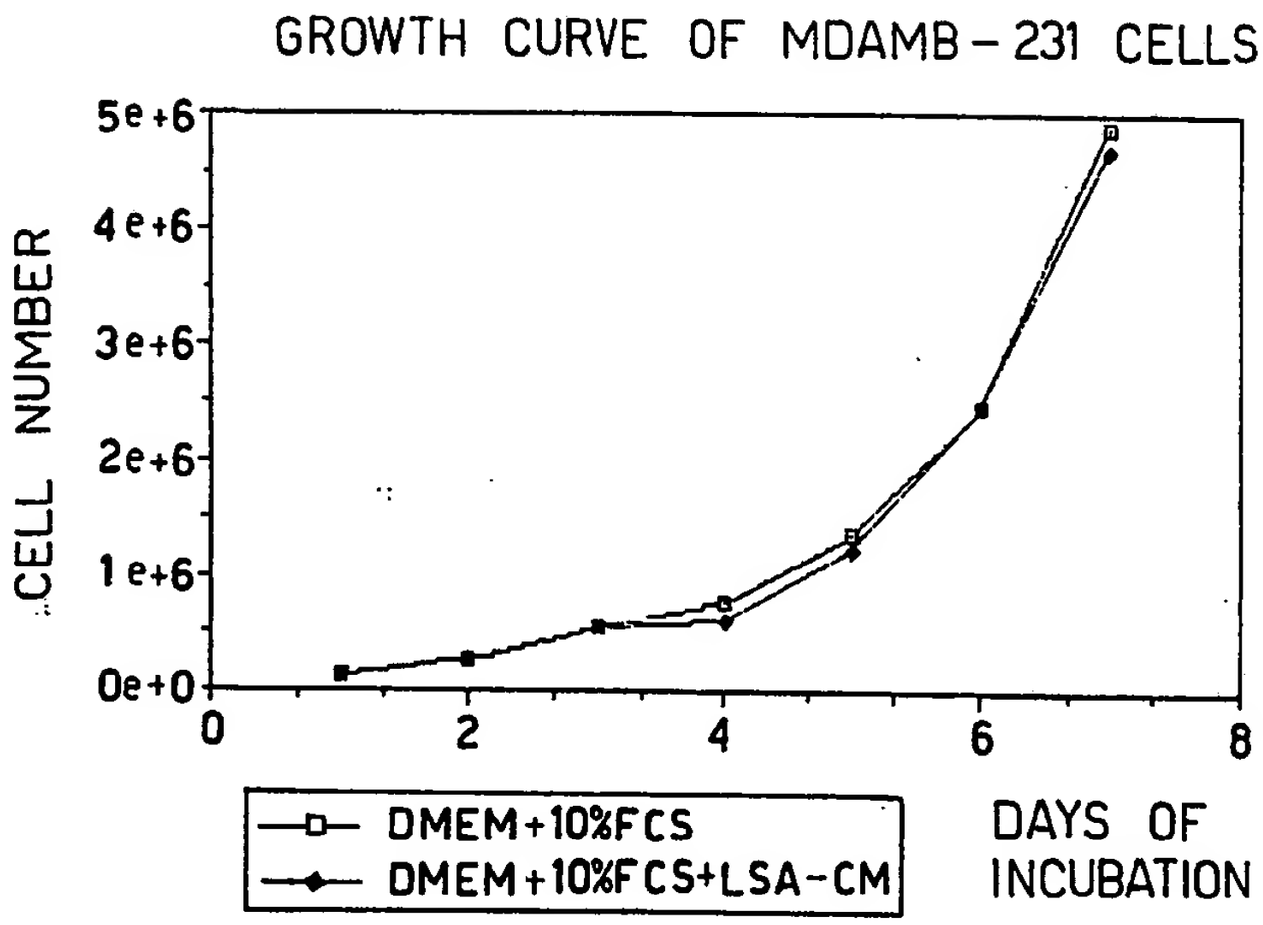
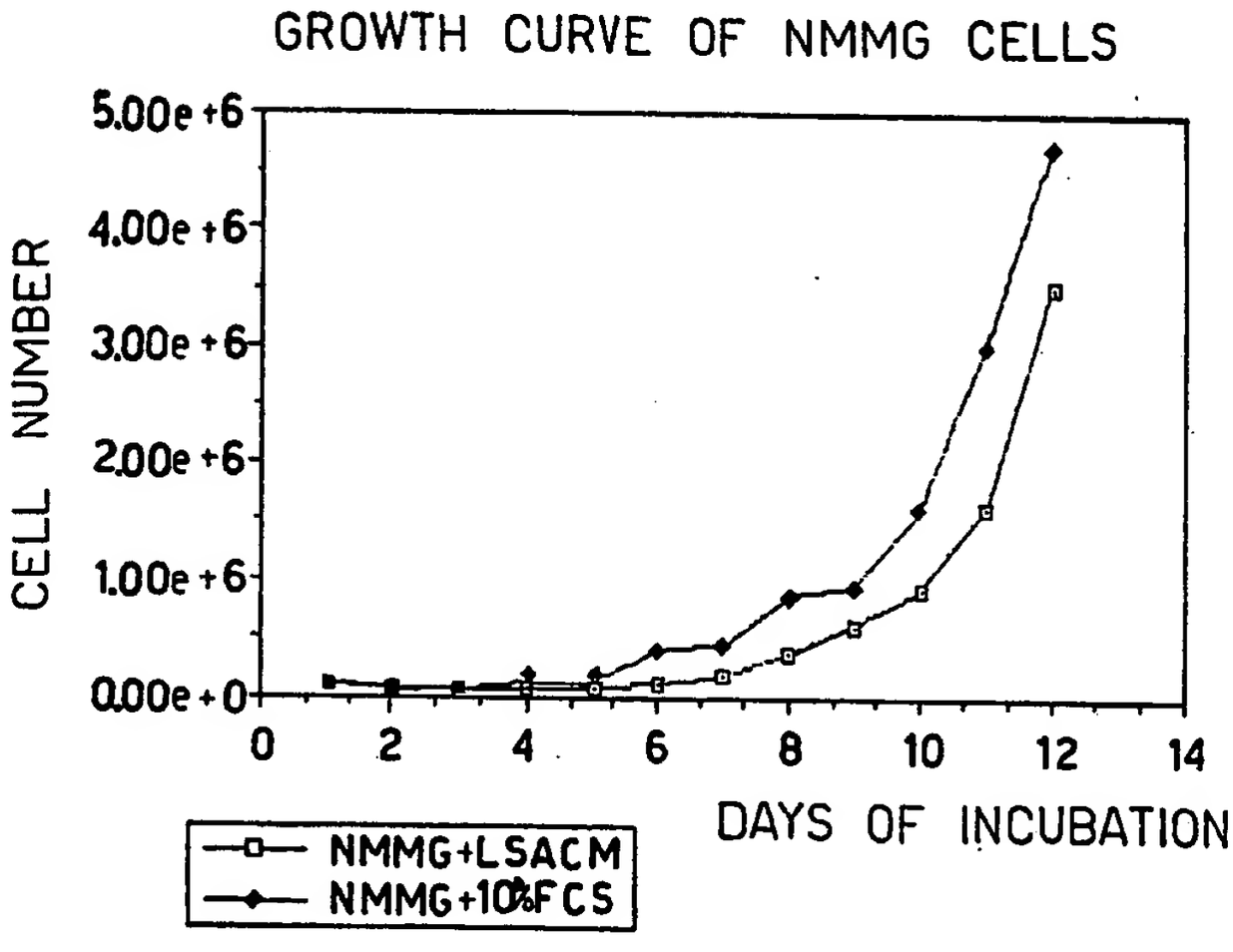

GROWTH CURVE OF MDAMB-231 CELLS
CELL NUMBER
5e+6
4e+6
3e+6
2e+6
1e+6
0e+0
0
2
4
6
8
DAYS OF INCUBATION
DMEM+10%FCS
DMEM+10%FCS+LSA-CM

FIG. 7


GROWTH CURVE OF NMMG CELLS
CELL NUMBER
5.00e+6
4.00e+6
3.00e+6
2.00e+6
1.00e+6
0.00e+0
0
2
4
6
8
10
12
14
DAYS OF INCUBATION
NMMG+LSACM
NMMG+10%FCS

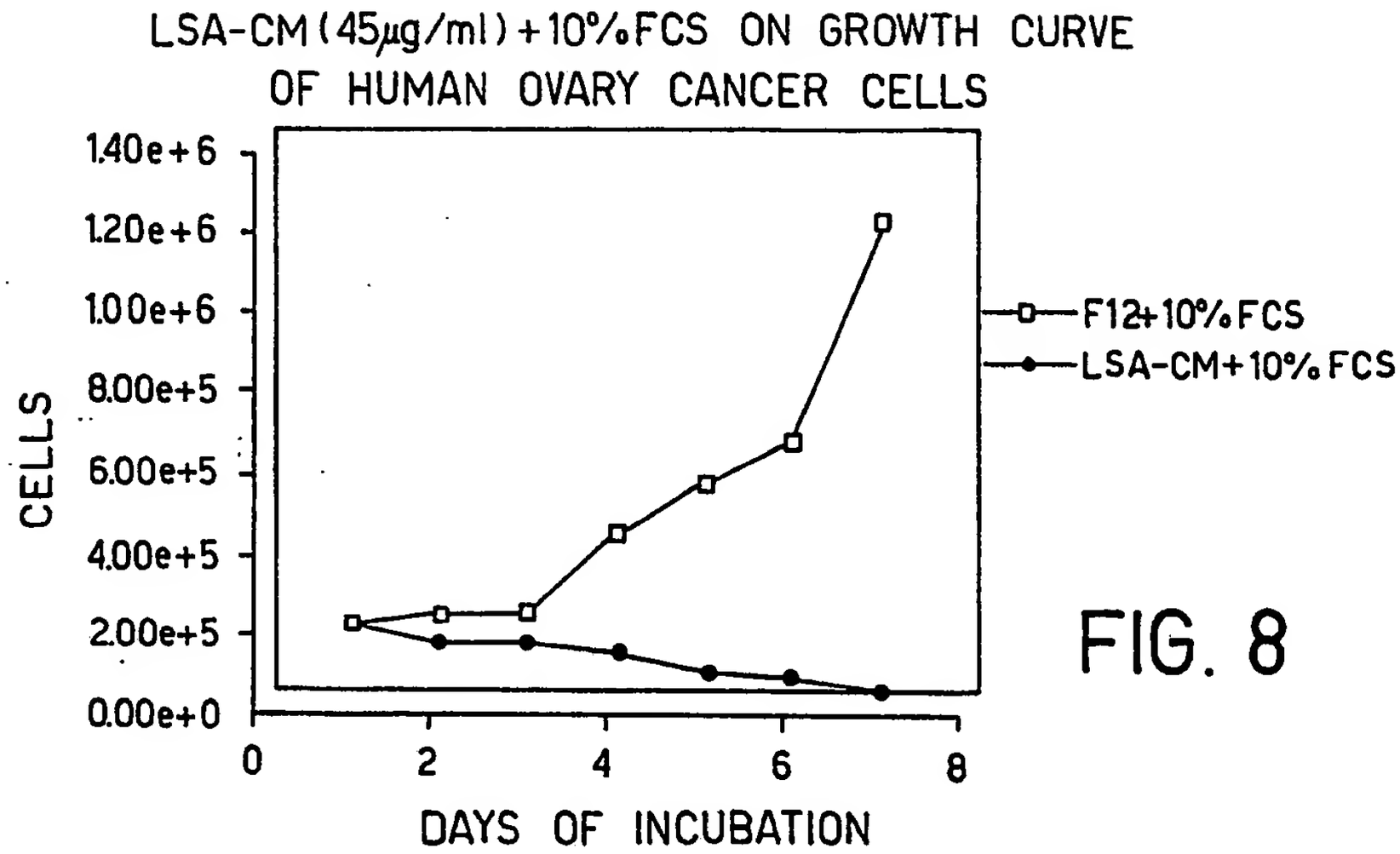
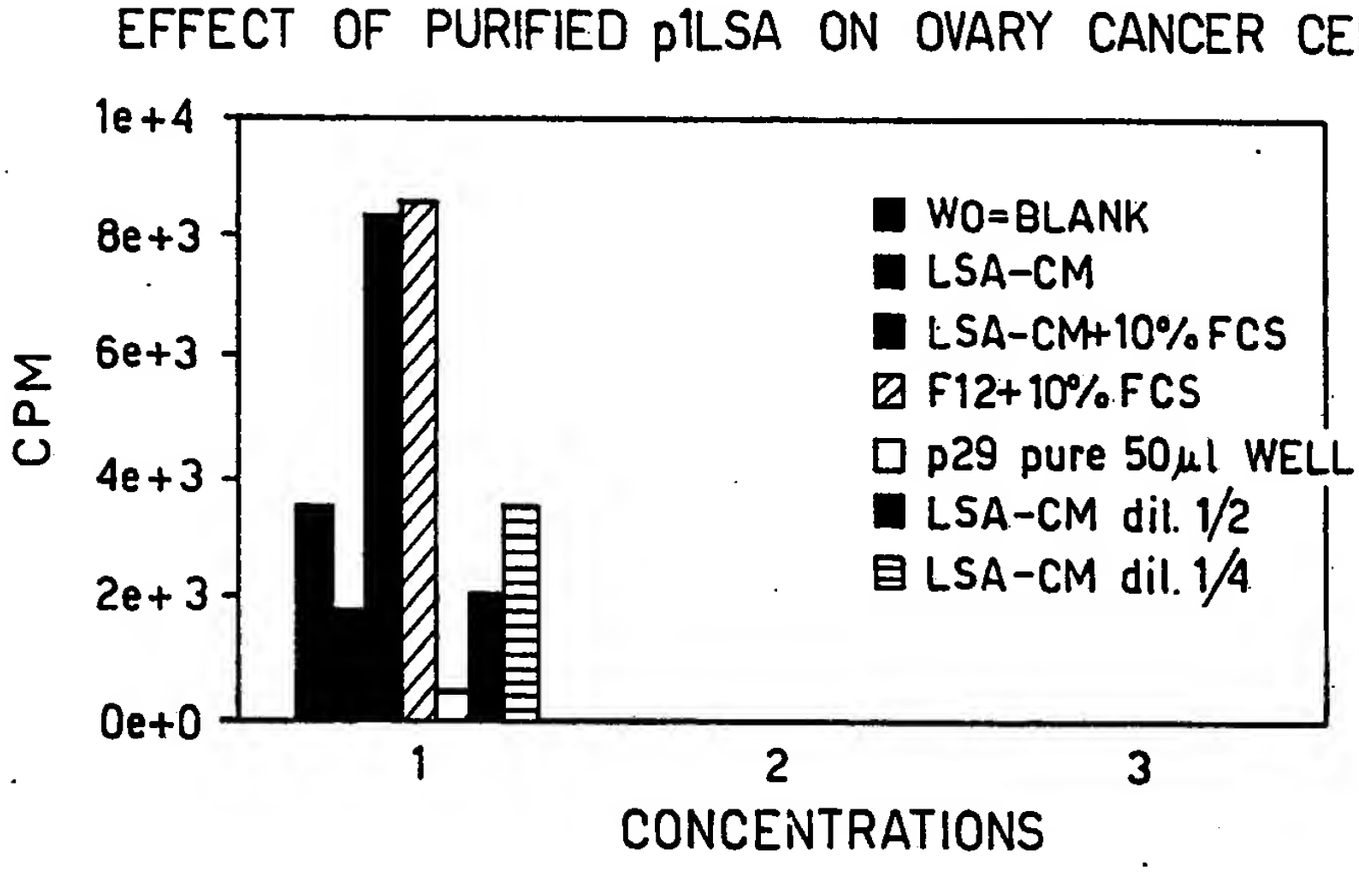

LSA-CM (45µg/ml) + 10% FCS ON GROWTH CURVE
OF HUMAN OVARY CANCER CELLS
CELLS
1.40e+6
1.20e+6
1.00e+6
8.00e+5
6.00e+5
4.00e+5
2.00e+5
0.00e+0
0
2
4
6
8
DAYS OF INCUBATION
F12+10% FCS
LSA-CM+10% FCS

FIG. 8

FIG. 9


EFFECT OF PURIFIED p1LSA ON OVARY CANCER CELLS
CPM
1e+4
8e+3
6e+3
4e+3
2e+3
0e+0
1
2
3
CONCENTRATIONS
WO=BLANK
LSA-CM
LSA-CM+10% FCS
F12+10% FCS
p29 pure 50µl WELL
LSA-CM dil. 1/2
LSA-CM dil. 1/4

INTERNATIONAL SEARCH REPORT

International Application No PCT/IT 93/00020

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/12; C07K13/00; A61K37/02; C12N5/06
C12N5/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	NUCLEIC ACIDS RESEARCH. vol. 14, no. 10, 27 May 1986, ARLINGTON, VIRGINIA US pages 4127 - 4145 EILEEN HICKEY ET AL. 'Sequence and organization of genes encoding the human 27 kDa heat shock protein' cited in the application see page 4128, paragraph 4 - page 4129; paragraph 1 see page 4131, paragraph 3 - page 4134, paragraph 1; figure 2A --- -/--	1-3,8-10

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29 JUNE 1993	13. 07. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MONTERO LOPEZ B.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	CANCER RESEARCH vol. 51, no. 19, 1 October 1991, pages 5245 - 5252 JACQUES HUOT ET AL. 'Increased survival after treatments with anticancer agents of chinese hamster cells expressing the human Mr 27,000 heat shock protein' see page 5246, left column, paragraph 2 see page 5249, right column, paragraph 1 - page 5251, left column, paragraph 2 -----	11